NUTRITIONAL STUDIES ON THE AMOEBO–FLAGELLATE, TETRAMITUS ROSTRATUS[1]

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Nutritional studies on amoebae have lagged far behind those on ciliates and flagellates. The earliest controlled work on the nutrition of members of the Sarcodina concerned the cultivation of single species of amoebae in the presence of bacterial mixtures. Here, most emphasis was placed upon the culture medium itself, the living bacteria being considered as obligate factors for growth of the phagotrophic forms. Scattered reports of axenic (Dougherty, 1953) cultures of amoebae have been recorded in the literature, but the earliest accounts have not been corroborated by other investigators. Included in these is the reported cultivation of some amoebae *in vitro* on sterile mammalian tissues by Williams (1911). She believed that these amoebae were parasites, but this is doubtful in the light of present research upon known parasitic forms. Oehler (1924) claims to have grown several unknown species of free-living amoebae under axenic conditions upon water-agar containing coagulated serum. As far as is known this work has not been re-investigated.

One of the first species to be grown free of living bacteria and which can be obtained today is the free-living amoeba, *Acanthamoeba castellanii.* Castellani (1930) found that this amoeba was capable of growing upon a killed bacterium and dead yeast. Cailleau (1933b) succeeded in obtaining an entirely liquid medium for *A. castellanii* consisting of peptone and added salts. This has been modified somewhat by Storm, Hunter and Cowperthwaite (1951) who grew the amoeba upon a medium containing acid-hydrolysate of gelatin and a low concentration of skim milk. The same authors (1951) have also reported growth of *Hartmannella rhysodes* upon an autoclavable liquid medium containing hydrolysates of gelatin and free oleate esters.

Reich (1935) reported axenic cultivation of the soil amoeba, *Mayorella palestinensis,* upon a clear medium consisting of peptone, a balanced salt solution, and added dextrose. The latter component appeared indispensable for good growth.

On the other hand, many investigators have failed to grow amoebae axenically. Among these is Wherry (1913) who, in preliminary experiments, could not succeed in growing an amoebo-flagellate (probably *Naegleria gruberi*) free of the bacterial flora. Rice (1935) succeeded in growing the marine amoeba, *Flabellula mira,* in monobacterial cultures, but failed to cultivate this form axenically by adding either killed bacteria or various amino acids to the basal medium.

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Although a few of the parasitic flagellates have been cultured axenically *in vitro,* the nutrition of the parasitic amoebae has provided a more difficult problem. In the case of *Entamoeba histolytica,* the amoebae apparently require not only certain metabolites provided by the basal medium but also an anaerobic environment mediated by the associated bacteria (Snyder and Meleney, 1943), as well as the bacterial bodies themselves (Shaffer, 1952). As far as is known at present, *E. histolytica* has not been grown *in vitro* free of the influence of living protoplasm (other Protozoa, bacteria, embryonic tissue cultures).

As one approach to the nutrition of the parasitic amoebae it seemed desirable to investigate a free-living coprophilic form, since these Protozoa pass sporadically through the lower digestive tract of various animals. Although sharing the same environment as intestinal parasites in this respect, they seem to be incidental guests of their hosts and might conceivably stand at the threshold of parasitism. Little work has been done on the amoebo-flagellate, *Tetramitus rostratus,* apart from morphological and cytological studies. According to most investigators this organism is a coprophile which actively feeds and multiplies in its trophic stages upon substrates rich in organic material. It has also been found in fecal cultures of various animals by Bunting (1922) and Rafalko (1951). In view of these facts this organism provided an excellent opportunity for a nutritional investigation which might link free-living and parasitic forms.

Materials and Methods

The strain of *Tetramitus* used in this investigation was obtained in December, 1950 by Dr. William Balamuth from Miss Lois Norman at the Communicable Disease Center in Chamblee, Georgia. It had been found in a "sterile" urine specimen in Austin, Texas in the same year. Routine cultures of the wild stock were maintained at room temperature upon a medium consisting of 0.5% Difco yeast extract plus 0.5% Difco peptone (YP) made up in distilled water. The diphasic nature of this organism was first demonstrated by Bunting (1922, 1926), who pointed out a reversible amoeba-to-flagellate transformation in its life cycle. Although both stages of the present strain appeared in the bacterized maintenance broth only the amoeboid form was present on solid agar-containing media or under axenic conditions.

Handling of cultures

In order to obtain large numbers of bacteria-free amoebae for use in nutritional studies it was thought advisable to establish the trophozoites in a two-membered culture with a penicillin-sensitive bacterium. Washed cysts from the wild stock of *Tetramitus* were first sterilized by a series of chemical procedures. These included successive treatment at 23° C. with 1 : 50,000 $HgCl_2$ for one hour and with 1 : 5000 $KMnO_4$ for 30 minutes. The cysts were then implanted into a tube of YP broth containing *Micrococcus pyogenes* var. *aureus.* Bacteria-free amoebae could be obtained from this culture by inoculating 6- to 14-day-old trophozoites onto plates of YP medium containing 1.5% agar plus 500 units/ml. of penicillin and incubating them for 3 days at room temperature. Their sterility was routinely tested by inoculating them into Difco fluid thioglycollate medium and Difco stock culture agar (SCA).

In assaying prepared media the general plan was as follows: Penicillin-treated amoebae were gently flushed off the agar surface with sterile tap water and pooled in a test tube. The organisms were inoculated in 0.1-ml. amounts into 150 × 18 mm. cotton-stoppered tubes containing 5 ml. of sterile test broth. The viability of the treated amoebae used in each experiment was tested by inoculating them into broth containing living bacteria. All cultures were incubated in a moist chamber at 30° C. in a slanting position and observed at regular 2- to 4-day intervals for as long as 10 to 14 days. Transplants were made in approximately 0.2-ml. amounts. In order to eliminate carry-overs consideration was given only to the fourth subculture when evaluating positive results. Positive cultures were always re-checked for bacterial sterility in fluid thioglycollate medium and SCA.

As required in population runs, amoebae from broth cultures were counted upon four hemocytometer fields and the results averaged.

Preparation of media

In axenic assays, initial experiments were designed to modify the concentration of components of the original YP medium. In some cases substitution for the yeast extract was made with Anheuser-Busch autolyzed or pepsin-digested yeast. Protein digests including BBL trypticase, BBL phytone, Difco proteose-peptone and Difco tryptone in concentrations from 0.1% to 3.0% were substituted for the peptone fraction. More complex media involved the addition of Cerophyl (dehydrated cereal grass leaves), liver extract, cream, whole egg, blood and selected vitamins to the basic YP medium in varying concentrations.

A review of the literature suggested types of media which have been used to grow Protozoa axenically. Pressed-yeast juice was prepared according to the method of Johnson and Baker (1942), in which they cultivated *Paramecium multimicronucleatum*. Variations of their medium, sterilized by Selas filtration, consisted of adding the concentrated juice to distilled water in amounts ranging from 3.0% to 50.0% in approximately two-fold concentrations. Cailleau (1933a, 1933b) reported growth of *A. castellanii* upon two kinds of media. Her first medium (1933a) and variations of her later medium (1933b) were tried. Trypticase, phytone and peptone in concentrations from 1.0% to 3.0% were substituted for the peptone fraction in her newer medium. Reich's medium (1935) for *M. palestinensis* was also utilized in the screening procedures.

All media except Johnson's and Baker's pressed-yeast juice were sterilized by autoclaving for 20 minutes at 15 pounds pressure. The hydrogen-ion concentrations were not critically controlled but were adjusted when possible to approximate neutrality.

Various species of dead bacteria were utilized as a food source in many of the experiments. The organisms were grown upon YP agar in large petri dishes for 24 to 48 hours. After maximum growth was obtained the cells were scraped off the plates, suspended in tubes of distilled water and autoclaved for 30 minutes at 15 pounds pressure.

Preparation of bacterial hydrolysates and extracts

It later became evident that certain substances present in the dead bacterial cells (particularly in *Bacillus cereus* and *B. subtilis*) were required for growth of the amoebae. The *B. cereus* cells were fractionated in the following manner: Thirty grams of freshly harvested bacteria were added to an equal weight of alumina and the aggregate ground by hand with dry ice. One hundred and sixty milliliters of distilled water were added to the crushed cells and the alumina centrifuged out. The suspended cells were divided among three beakers in equal amounts and enough 1 N HCl and 1 N NaOH added separately to two of the containers to make 0.1 N solutions of acid and base, respectively. The contents of the third beaker were adjusted to pH 7.00. All three of the vessels were autoclaved for 30 minutes at 15 pounds pressure yielding acid-, alkaline-, and neutral-hydrolyzed fractions. The contents from each beaker were then divided into two parts:

(a) One-half of the cell suspension from each beaker was subjected to dialysis through a Visking casing for 28 hours at 4° C. in liter beakers with four changes of cold distilled water. The dialysates were discarded for the purposes of these experiments. After dialysis the pH of the three non-dialyzable [3] fractions containing the cell-residues was adjusted to neutrality. The cell-residues were collected and washed and the non-dialyzable supernatants concentrated to 5 ml. by boiling.

(b) The pH of the remaining non-dialyzed acid, alkaline and neutral fractions was also adjusted to neutrality; the cell-residues were collected and the non-dialyzed supernatants concentrated in a similar manner.

All fractions were then re-autoclaved and stored at 4° C.

In later investigations it became necessary to extract the fat-soluble fractions of the neutral-hydrolyzed non-dialyzed supernatants with various fat solvents. This was accomplished by shaking each supernatant with an equal volume of solvent, collecting the latter and repeating the procedure several times. The solvent was evaporated to dryness and the sediment brought up to the original volume with distilled water. Before extracting with alcohol or acetone it was first necessary to evaporate the supernatants to dryness; the insoluble materials were then centrifuged out.

Vitamins, purines, pyrimidines and amino acids

In experiments requiring special nutrients, mixtures of vitamins, purines, pyrimidines and amino acids were added in place of certain bacterial fractions. A stock solution of vitamins was prepared consisting of 50 mg. each of choline·HCl, folic acid, inositol, nicotinic acid, paramino-benzoic acid, calcium pantothenate, pyridoxine·HCl, riboflavine, thiamine·HCl and 25 μg of biotin. The purine-pyrimidine mixture consisted of 50 mg. each of adenine sulfate, cytidylic acid, guanine, thymine, uracil and xanthine. Both of the above mixtures were separately suspended in 500 ml. of distilled water, filtered through No. 03 Selas filters and stored at 4° C. The amino acid stock mixture contained 50 mg. each of L-asparagine, DL-alanine, L-cystine, L-glutamic acid, DL-methionine and DL-tryptophane. The above acids were added to 50 ml. of distilled water and autoclaved for 10 minutes at 15 pounds pressure.

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[3] *Non-dialyzable* refers to a retained fraction after being subjected to dialysis, as opposed to *non-dialyzed* fractions which were not subjected to dialysis.

Experiments and Results

Selection of a medium

Experiments with most empirical formulae and those media already known to support axenic growth of the free-living Protozoa described above, proved unsuccessful for *Tetramitus*. Axenic growth of *Tetramitus* could be obtained, however, when washed autoclaved *Bacillus cereus* was added to a medium consisting of 0.5% yeast extract, 0.5% peptone and 1.0% Wilson liver concentrate (N.F.). In the yeast-peptone-liver medium (designated as YPL) sterile young amoebae were 2–3 times larger than normal (60 μ in diameter) and usually appeared opaque and immobile, but they became progressively more active as the cultures became older. Through varying the pH from 5.5 to 7.9 it was found that better growth could be obtained near neutrality (6.8–7.3). Altering the temperature from 20° C. to 35° C. demonstrated that optimum growth (150 per mm.^{3}) could be reached in approximately 12 days at 30° C.

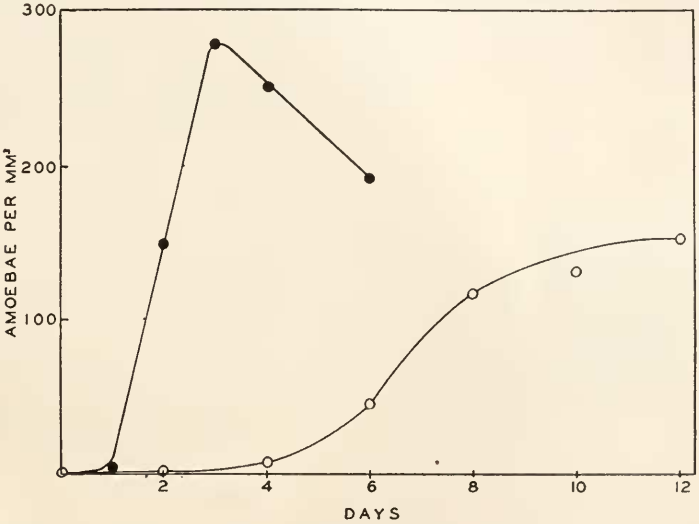

FIGURE 1. Comparison of growth of populations of *T. rostratus* with living vs. autoclaved *B. cereus* in 5 ml. of YPL medium at 30° C. Inoculum-= 870/ml. ●: Growth with living bacteria; o: Growth with dead bacteria. Note: Each point represents a separate tube.

A distinct lag phase was observed when the amoebae were grown with dead *B. cereus* (Fig. 1), which was considerably longer than that obtained with living bacteria. This would seem to indicate that although this medium is capable of supporting growth and reproduction of the amoebae, it is far from perfect and is incapable of supplying all the factors derived from cultures containing living bacteria. Preliminary experiments to shorten this lag with Selas-filtered, preconditioned bacterial cultures have not proved promising.

Attempts were made to substitute other autoclaved bacteria for *B. cereus* in YPL broth. Considerable growth of the amoebae could be obtained with killed *Escherichia coli, Neisseria catarrhalis, Sarcina lutea* and *Bacillus subtilis* by culturing at 8-day intervals. No growth could be obtained with autoclaved *Pseudomonas fluorescens.* Substitution of autoclaved yeast Harris, Anheuser-Busch and Difco whole yeast failed to replace this bacterial factor.

Although the liver portion of the medium could be reduced to 0.25% without appreciably affecting growth, its complete elimination resulted in poor numbers of amoebae. However, amoebae have been cultured in this medium (YP) for two months with the addition of large amounts of autoclaved *B. cereus* or *B. subtilis* at each sub-culture. The liver-deficient medium would not support growth of the amoebae with added autoclaved *E. coli* or *N. catarrhalis.* Apparently *B. cereus* and *B. subtilis* contain at least one or more factors found in liver extract which seem to be lacking in the two non-spore-forming bacteria. These factors remain to be elucidated.

TABLE I

Growth-supporting capacity of fractions of Bacillus cereus for Tetramitus rostratus in sterile yeast-peptone-liver medium

			Neutral hydrolysis		Acid hydrolysis		Alkaline hydrolysis	
			Non-dialyzed super-natant	Non-dialyzable super-natant	Non-dialyzed super-natant	Non-dialyzable super-natant	Non-dialyzed super-natant	Non-dialyzable super-natant
			−	−	−	−	−	−
Neutral hydrolysis	Non-dialyzed cell-residue	* −						
	Non-dialyzable cell-residue	−	+	−	−		−	
Acid hydrolysis	Non-dialyzed cell-residue	+						
	Non-dialyzable cell-residue	+			+			
Alkaline hydrolysis	Non-dialyzed cell-residue	−	* −				−	
	Non-dialyzable cell-residue	−						−

* Present only in initial culture.

+ Growth of amoebae.

− No growth of amoebae.

Note: All supernatants were added as 1 pt. to 4 pts. of YPL medium. Cells were added in excess (approx. 0.1 ml. wet mass).

Bacterial hydrolysates

Since something in the bacterial cell appeared necessary for sustained growth of *Tetramitus* in YPL medium, partial hydrolysis of *B. cereus* was carried out as described, in order to separate possible essential fractions. It was found at the outset that the substance(s) survived autoclaving for as long as 50 minutes, and a thorough washing of the intact cells failed to remove any activity.

It can be seen from Table I that amoebic growth resulted in YPL medium with the addition of the neutral-hydrolyzed non-dialyzable cell-residue to the neutral-hydrolyzed non-dialyzed supernatant. Although the non-dialyzed supernatant was routinely added as one part to four parts of YPL medium (by choice), it was found that it would maintain trophic growth in one-half this amount as long as the cell-residue was added. No growth could be obtained using the supernatant alone even when raised to 50% of the total medium. It can also be seen that the non-dialyzable supernatant is completely inactive whether used alone or with the non-dialyzable cell-residue. This residue still retained a small quantity of fat which was demonstrated with the Sudan Black B stain of Burdon (1946). The results obtained with the neutral-hydrolyzed fractions, therefore, would seem to indicate that at least two factors from the bacterial cell are required for growth of the amoebae: one dialyzable found in the bacterial supernatant and the other non-dialyzable found in the cell-residue.

Macerated cells treated with 0.1 N HCl and then subjected to dialysis retained their activity. Microscopical examination of the cells revealed mostly disintegrated cell husks with some fat retained in the debris. The non-dialyzed supernatant from these cells (Table I) was inactive when utilized with the neutral-hydrolyzed non-dialyzable cell-residue.

Alkaline hydrolysis of *B. cereus* seemed to destroy more than one factor required for growth of *Tetramitus.* Combinations of alkaline-hydrolyzed fractions together or with neutral-hydrolyzed fractions were inactive. Examination of the cell fragments revealed the absence of fat. When alkaline-treated fractions were added with normal autoclaved whole cells growth ensued, demonstrating that there is no toxicity factor involved here.

1. *Preliminary attempts to replace the dialyzable fraction*

Utilizing the lead obtained from neutral hydrolysis, experiments were performed to discover the nature of the factor(s) which were removed by dialysis. In these experiments the non-dialyzable fraction was provided by the non-dialyzable cell-residue. The possible dialyzable factors were sought separately in a mixture of vitamins, a mixture of purines and pyrimidines and amino acids. The vitamin mixture described above added in 0.002-ml., 0.02-ml. and 0.2-ml. amounts per 5 ml. of YPL medium gave no indication of supporting growth. The mixture of purines and pyrimidines added in 0.02-ml., 0.2-ml. and 2.0-ml. amounts to the vitamin-containing medium also failed to maintain growth. Mixed amino acids added in 0.25-ml., 0.5-ml. and 1.0-ml. amounts in combination with the vitamins and the purine-pyrimidine mixture gave no promising results.

In view of the fact that the substituted mixtures showed no activity, it was postulated that non-dialyzable fractions in the bacterial supernatant were also required for growth. This immediately suggested lipoidal material. To test this possibility, the non-dialyzable supernatant was utilized with the non-dialyzable cell residue as non-dialyzable fractions. No growth of the amoebae resulted with the addition of the vitamin mixture to these fractions in YPL medium. This was re-investigated with the lipoid-extracted portions of the supernatant added as one part to four parts of YPL medium. In no case was growth of *Tetramitus* observed when either benzene-, alcohol-, ether-, or acetone-extracted supernatant was added to the vitaminized medium containing the non-dialyzable cell-residue.

2. Preliminary attempts to replace the non-dialyzable fraction

In routine investigation of the non-dialyzable fraction, neutral-hydrolyzed non-dialyzed supernatant was added to every tube of YPL broth as one part to four parts of medium; and in addition the vitamin mixture was added in a concentration of 0.1 ml. per 5 ml. of broth.

In order to eliminate the possibility that an essential metal might be tied up in the bacterial residue, the cells were completely ashed by flaming them in a Pyrex tube and then added to the culture medium. No growth of *Tetramitus* could be obtained. The separate addition in 1.0% and 5.0% proportions of peptone, tryptone, tryptose, proteose-peptone, yeast extract, trypticase and phytone failed to replace this factor. Skim milk in concentrations of 0.01% to 0.5% has also failed. Experiments are planned to replace this factor with known proteins and polysaccharides.

DISCUSSION

Although only preliminary experiments have been carried out on the nutritional requirements of *Tetramitus,* it has been shown that the dead bacterial cell provides some essential constituent(s) for axenic growth of the amoebae in the yeast-peptone-liver medium. The types of killed bacteria it can use in this medium are non-specific since it has utilized gram-negative and gram-positive representatives of the cocci group, gram-positive spore-formers and a gram-negative coliform. It appears, however, that although *Tetramitus* will grow in YPL medium with most of the species of autoclaved bacteria investigated, only the spore-formers were utilized by the amoebae in the liver-deficient medium. Since *Bacillus cereus* and *B. subtilis* contained large quantities of fat, it is interesting to speculate whether they provide certain lipoidal substances present in the liver extract. This hypothesis should be tested by adding to the YP medium the fat-extracted portions of these cells together with the autoclaved non-spore-forming bacteria.

The finding that *Tetramitus* can utilize certain heat-stable metabolites found in microorganisms is not a new one when considering Protozoa in general. Johnson (1936) was able to obtain growth of the holotrichous ciliate, *Glaucoma ficaria,* in suspensions of 11 species of dead bacteria as well as 6 species of dead flagellates, using a balanced salt solution as a basal medium. Glaser and Coria (1935) established *Paramecium caudatum* and *P. multimicronucleatum* as well as other Protozoa upon a medium containing dead yeast cells as an indispensable nutrient. Van Wagtendonk and Hackett (1949) secured axenic growth of *Paramecium aurelia* but had to provide a 24-hour-preconditioned, autoclaved lettuce infusion culture of *A. aerogenes* with autolyzed yeast. When either component was omitted growth of the ciliates stopped, indicating essential substances other than the bacterial fraction.

It is evident from the preliminary work on *Tetramitus* that this organism is not as fastidious in its growth requirements as the parasitic amoebae. The fact that it can grow and multiply aerobically in the absence of other living protoplasm tends to make the nutritional approach an easier one. With *Entamoeba histolytica*, on the other hand, nutritional studies have been hampered by its dependency upon associated living organisms and upon its extreme sensitivity to oxygen. Recently a certain substance(s) in dead bacteria has also proved essential for this latter species. Karlsson, James and Anderson (1952) have shown that when an autoclaved culture of a streptobacillus in liver-proteose-peptone medium was used as a substrate for *E. histolytica* under antibiotic treatment to suppress bacterial growth, fair growth of the amoebae resulted. Use of filtered media resulted in the loss of activity, suggesting the active material was present in the cells. Later Karlsson (1952) showed that 90% of the cells' activity was destroyed during the first 20 minutes of autoclaving. In the present work on *Tetramitus* the bacterial fractions are clearly heat-stable, since prolonged autoclaving for 50 minutes does not seem to destroy their activity. The streptobacillus factor for *Entamoeba* was completely destroyed by 0.1 N NaOH in 5 minutes at room temperature but could withstand treatment with 0.1 N HCl for one hour, suggesting similarity to those factors found in the *B. cereus* cell. It is also interesting to note that Karlsson's streptobacillus fraction could not be extracted with fat solvents.

Although no definite decision can as yet be made, the dialyzable factor in the cell-extracted supernatant of *B. cereus* suggests some protein fragment, for example, a polypeptide or some other dialyzable substance of relatively low molecular weight. There exists the possibility that several essential substances, both dialyzable and non-dialyzable, may be present in the supernatant. Collection and analysis of the dialysates would be of value in elucidating these fractions. The supernatant fraction appears to remain with the cell-residue when subjected to acid hydrolysis (Table I). Further evidence for this was shown by complete inactivity of the acid-hydrolyzed non-dialyzed supernatant. Preliminary experiments have shown that this acid fraction contained no inhibiting substance when added to acid-hydrolyzed cells.

The non-dialyzable fraction in the cell residue would suggest substances either proteinaceous or polysaccharide in nature. More complete analysis of this fraction is required before arriving at any conclusions.

Summary

1. *Tetramitus* was cultivated indefinitely under axenic conditions upon a medium consisting of 0.5% Difco yeast extract, 0.5% Difco peptone and 1.0% Wilson liver concentrate (N.F.) with selected types of autoclaved bacteria.

2. The liver concentrate could be eliminated with subsequent reduction in trophic growth, only if killed *B. cereus* or *B. subtilis* was utilized as the bacterial fraction. Other bacteria (*Neisseria catarrhalis, Escherichia coli*) could not be substituted for these spore-formers in this medium.

3. It was found that the *B. cereus* cell contained at least two heat-stable fractions necessary for growth; neutral hydrolysis yielded a non-dialyzable fraction found in the cell-residue and a dialyzable fraction found in the bacterial cell supernatant.

4. Both factors were stable to autoclaving with 0.1 N HCl for 30 minutes at

15 pounds pressure and were retained in the acid-treated cell-residue. Alkaline hydrolysis destroyed activity of all the fractions.

5. Preliminary attempts have failed to substitute for the factors found in the neutral-hydrolyzed bacterial supernatant by employing selected vitamins, amino acids, purines and pyrimidines.

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